THE DETERMINATION OF RESIDUAL MOISTURE IN DRY BIOLOGICAL SUBSTANCES

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During the past few years, the extreme stability of biological products in dry form has been much more generally recognized and made use of. Various processes of gentle desiccation have been developed; those of Craigie (1), Elser *et al.* (2), Reichel (3), and Flosdorf and Mudd (4) utilize the preferable procedure of drying from the frozen state.

The manner of drying (and of freezing if from the frozen state (3)) affects the degree of stability, solubility, and of extractability with lipid solvents without denaturation. Whatever the method used, however, the stability is influenced to a major extent by the degree of dryness attained. The residual moisture content of lyophile[1] biologically active serum products should be below 2 per cent and preferably in the range of 0.5 to 1.0 per cent (5). This low range has required a more rigid method for analysis than any previously available.

Benedict and Manning (6) as early as 1905 pointed out that the analysis for moisture in biological substances low in moisture content cannot be carried out by direct determination. Fats and other substances are lost in the oven at 110° and physical and chemical changes occur which affect the weight. In order to observe the loss in weight of water under conditions which allow no other changes to occur, vacuum desiccation (1.0 mm. of Hg) over sulfuric acid was recommended. Sulfuric acid, however,

[1] Lyophile has been applied by Reichel (3) to designate that desiccation has been carried out rapidly from the frozen state after initial rapid freezing at −50° or lower.

has an aqueous tension at room temperature greater than that of the desiccants usually used in drying biologics, including dry ice condensers. Benedict and Manning predicted that use of a high vacuum at 50–60° might be the method of choice for biological substances, but that at that time there existed no easy technical method or apparatus for combining that degree of heat with the high vacuum.

Porcher (7) recommended P_2O_5 desiccation at 45° for a period of 48 to 72 hours for dried milk, whereas Holm (8) suggested heating for 1 hour at 100° and 100 mm. of Hg. Kerstow (9) found that 3 to 4 hours were necessary for complete elimination of the water by Holm's method. The official methods of analysis of the Association of Official Agricultural Chemists (10) for milk and for other biological products call for vacuum oven desiccation (100 mm. of Hg) at temperatures ranging from 70–100°, depending upon the product. We have accordingly investigated the influence of time and temperature of heating on the determination of the residual moisture content of gently dried sera in order to develop a satisfactory method for our purposes.

EXPERIMENTAL

To devise a method for determination of the residual moisture content, it is necessary to have a standard of accuracy. By using desiccation at room temperature over P_2O_5 for this purpose, we have avoided danger of the losses in weight from the sources pointed out by Benedict and Manning, yet accurate values have been obtainable, particularly when attainment of equilibrium is accelerated under high vacuum, because P_2O_5 is the classical desiccant having an immeasurably low aqueous tension. For a standard of accuracy, it was desirable not to use a temperature even as high as that used by Porcher, as it is known that changes in delicate immunological systems occur even at relatively low temperatures. As a routine procedure, however, this method is too slow and too much open to error because of the excessive manipulation required. The varying amount of moisture in the unknown and other factors make the length of the desiccation period uncertain. It was necessary, therefore, to weigh after a number of desiccating periods, with the surface of the P_2O_5 freshly scraped each time, until the samples attained a constant weight.

The test materials were sera of various species and other substances. We have found that the total time which is required to reach constant weight over P_2O_5 *in vacuo* varies from 48 to 144 hours for 1 gm. samples containing initially 1.0 to 25.0 per cent moisture. Desiccation carried out over periods of 2 to 3 weeks indicates that a constant weight is actually attained. The first experiments were designed to determine the effect of heating at 110°. The results appear in Table I and are the average results from analyses carried out in triplicate.

TABLE I

Apparent Residual Moisture Content of Lyophile Serum As Indicated by 110° Oven Compared with P_2O_5-Vacuum Method

Material	Sample No.	P_2O_5 results Control	Oven results, 110°						
			2 hrs.	3 hrs.	4½ hrs.	20 hrs.	48 hrs.	90 hrs.	2 wks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Pool of guinea pig serum (100 animals)	1	24.8		24.8	24.9	25.0	25.0		24.9
	2	1.0	2.6	2.6	2.7	2.9	3.0	3.2	
	3	0.5		0.7	1.2	1.5	1.5		2.0
Pool of rabbit serum (4 animals)	4	0.9	1.6	1.6	1.7	1.8	1.9	2.1	
Pool of human serum (80 persons)	5	0.9	1.3	1.3	1.4	1.3	1.4	1.4	
Horse serum (normal)	6	4.3		4.5	4.5	4.6	4.7		5.4
	7	1.5	1.0	1.9	2.0	2.1	2.2	2.4	
	8	0.2		0.5	0.5	0.8	0.7		1.1
Horse globulin (diphtheria antitoxin)	9	0.2		0.1		0.3	0.3		0.6

On the basis that lyophile serum, after completion of its desiccation by means of P_2O_5, can be heated at 110° for 10 to 18 hours without additional loss in weight, it had been believed that the oven at 110° could be used equally well in moisture determinations (4). The results in Table I, however, indicate that serum not dried over P_2O_5, but with 0.5 to 1.0 per cent moisture, loses weight in the oven at 110° even though the lyophile serum desiccated with P_2O_5 does not suffer such loss. Furthermore, the degree of loss is greater, for some unknown reason, in the desired stability range of 0.5 to 1.0 per cent residual moisture than it is at very high levels. The largest errors have arisen with guinea pig serum.

The slighest degree of decomposition or other weight loss occurring at 110° causes a large percentage error. A 10 ml. serum sample contains normally from 0.6 to 1.0 gm. of solids. Lyophile serum, containing 0.04 per cent of the original water, has a moisture content of about 0.5 per cent as expressed in terms of the dry solids remaining (P_2O_5-vacuum method). The actual weight of this moisture in the quantity of dry material mentioned above is 3 to 5 mg. Decomposition to the extent of 1 mg. would cause an error of 20 to 30 per cent. The error occasionally has been found to be as much as 300 per cent.

The vacuum oven at a relatively low temperature appeared to offer the best promise of meeting the requirements for a method of analysis, and in the present work the relation between time and temperature of heating at 10° intervals (50–100°) in a high vacuum oven has been investigated. This relation was compared with results obtained by the P_2O_5-vacuum method. Altogether 650 determinations have been made in this investigation, usually in triplicate. In comparisons of different methods the samples were all taken, in the case of any one substance, from a single container. The substance was powdered thoroughly by shaking vigorously in the sealed evacuated container and, while still sealed in the container, it was stored for several days at 37°. The material was considered then to be uniform in moisture content in all parts of the container. The weighing bottles used in the analysis were filled as rapidly as possible after the serum container was opened and numbered in the order of filling. Alternate samples were used for each method. The results obtained indicate that transference of the extremely hygroscopic samples can be accomplished without significant increase in moisture content.

It was found that 50° is the maximum temperature at which serum may be heated without decomposition of the more labile species; at this but not at lower temperatures moisture is completely eliminated within a reasonable interval of time. A constant weight could not be obtained generally at a higher temperature. Some species of serum may be heated as high as 80° without decomposition, but it is desirable to use a temperature that is applicable to all products of a generally similar nature. Typical results of the average of duplicate and triplicate determinations carried out in the vacuum oven are summarized in Table II.

It has been found that even at 50° if some sera be heated unnecessarily long, decomposition eventually will occur. 22 hours are ample for the sera even with higher moisture contents to attain constant weight; however, marked decomposition and further loss in weight do not become appreciable until after 48 hours. This permits sufficient latitude for a practicable general procedure to be set up. There is some variation with the sera from different species, but the degree of accuracy attainable with all types of serum heated at this one temperature is less than a ±15 per cent error, which is satisfactory. The use of a desiccation chamber, filled with P_2O_5 or drierite, in the oven-vacuum system (or with

TABLE II

Apparent Residual Moisture Content of Lyophile Serum As Indicated by 22 Hours Heating in Vacuum Oven at 50°, Compared with P_2O_5-Vacuum Method

Species of serum	P_2O_5	Vacuum oven
	per cent	*per cent*
Horse serum (normal)	2.34	2.27
Guinea pig serum	1.32	1.35
" " "	7.11	6.84
Horse globulin (diphtheria)	0.74	0.64
" " "	1.61	1.38
" serum (normal)	0.97	0.80
" " "	5.49	5.68
Rabbit " "	0.95	1.07
" " "	4.01	3.90

P_2O_5 in the oven itself) produced no difference in the final results. This is explained by the fact that the pump has sufficient capacity, even at a pressure of 0.1 mm. of Hg, for the amount of water vapor which is yielded by products of such low moisture content.

Determinations also were carried out in the oven at 50° but at atmospheric pressure and in a P_2O_5 desiccator at atmospheric pressure. In both cases it was clearly evident, from a comparison with results by the P_2O_5-vacuum method, that a vacuum is necessary in either the oven at 50° or in the desiccator with P_2O_5 (although over P_2O_5 the final equilibrium value would doubtlessly be established if sufficient time were allowed to elapse). The average of results obtained in triplicate are shown in Table III.

In operation of the P_2O_5-vacuum method, the practise has been

to evacuate the desiccators to a pressure of 0.05 mm. of Hg and then to close the stop-cock and shut off the pump. Tests showed that the desiccators hold their vacuum satisfactorily for many days. In the case of the vacuum oven, with which it is much more difficult to exclude completely all leaks and where there is no desiccant to absorb the water vapor, it is necessary to run the pump continuously. The directions in detail for the vacuum oven procedure based on these findings are given below.

For use with samples of approximately 1 gm. (the solids from 10 ml. of serum), low form, flat bottom weighing bottles (50 mm. diameter) with well ground stoppers[2] are used. These are pre-

TABLE III

Apparent Residual Moisture of Lyophile Normal Horse Serum As Indicated by Oven at 50°, Atmospheric Pressure, and by P_2O_5 Atmospheric Desiccation, Compared with Usual P_2O_5-Vacuum Method

Period of desiccation	P_2O_5-vacuum	Atmospheric pressure	
		By P_2O_5	Oven at 50°
hrs.	*per cent*	*per cent*	*per cent*
24	0.61	0.59	0
48	1.32	0.80	0.1
72	1.28	0.82	Increased in weight
98	1.31	0.82	" " "

pared by cleaning in chromic acid solution, followed by thorough rinsing with distilled water. The bottles with the lids tilted open are then placed in the oven[3] for 2 hours under a vacuum at 50°. The oven is connected directly to a Cenco Hyvac pump. The tubing should be short and all connections should be sufficiently tight to permit evacuation to 0.1 to 0.2 mm. of Hg (McLeod gage). The bottles with the lids closed are then removed from the oven and placed directly in a desiccator to cool. The desiccator should contain a large freshly scraped surface of P_2O_5. In 1 hour, temperature equilibrium is attained and the bottles are removed from the desiccator one by one and weighed to 0.1 mg. as quickly as possible.

[2] Arthur H. Thomas Company, catalogue No. 9965.

[3] Weber vacuum oven. Arthur H. Thomas Company, No. 7886 or No. 7888.

The bottles are now filled quickly with amounts of samples believed to be about 1 gm. each. The lids must be replaced on the bottles with as little delay as possible after transferring the sample.[4] The bottles are reweighed quickly in order to obtain the exact weight of the sample.

With the lids tilted, the bottles are placed in the vacuum oven regulated to 50° ± 1°. The lids should not be opened until after the bottles are placed in the oven; otherwise drafts may blow away small particles of the light and fluffy sample. The Cenco pump is turned on and allowed to run continuously for 22 hours with a pressure of 0.1 to 0.2 mm. of Hg. A pressure of 0.2 mm. of Hg should be reached within an hour or less.

In 22 hours the pump is shut off and air is slowly admitted to the oven. The precaution of admitting dry air into the hot oven has been found to be unnecessary. The lids are replaced instantly and the bottles removed to the desiccator for cooling. They are weighed after an hour to 0.1 mg. as previously.

By this procedure, any detectable errors which might arise from atmospheric conditions are avoided, even on days when the relative humidity approaches 100 per cent. This method is fully satisfactory for carrying out a large number of routine determinations and has been used in these laboratories in the analysis of over 1500 samples during the past year. It should be of general use in biological work.

BIBLIOGRAPHY

1. Craigie, J., *Brit. J. Exp. Path.*, **12**, 75 (1931).
2. Elser, W. J., Thomas, R. A., and Steffen, G. I., *J. Immunol.*, **28**, 433 (1935).
3. Reichel, J., U. S. patent 2,066,302, Dec. 29, 1936.
4. Flosdorf, E. W., and Mudd, S., *J. Immunol.*, **29**, 389 (1935).
5. Flosdorf, E. W., and Webster, G. W., to be published.
6. Benedict, F. J., and Manning, C. R., *Am. J. Physiol.*, **13**, 309 (1905); **18**, 213 (1907).
7. Porcher, C. H., *Ann. fals.*, **9**, 450 (1916).
8. Holm, G. E., *J. Assn. Off. Agric. Chem.*, **5**, 509 (1922).
9. Kerstow, J. T., *J. Assn. Off. Agric. Chem.*, **9**, 213 (1926).
10. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 4th edition (1935).

[4] It has been found that the moisture content will be increased by as much as 1 per cent of the weight of the solids in 1 minute of standing with the lid off (relative humidity of 68 per cent).